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Micro-homology mediated PCR targeting in *Saccharomyces cerevisiae*

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In the present study, we determined the amount of homology required for targeted integration of DNA fragments into the yeast genome. The procedure described here facilitates the manipulation of the yeast genome and eliminates the need to clone sequences homologous to a target site. In addition, this method is useful for applications in which only limited sequence information of the target is available. The procedure comprises of: (i) production of PCR primers to amplify a selectable marker containing flanking homology to the target of choice; (ii) transformation of yeast cells and (iii) selection of integrants.

Since the first development of this technique (1) different groups reported versions of this technique (2–4). However, the minimum length of homology required for targeted integration and the exact efficiencies of targeting as a function of homology length has not to date been determined.

To determine the minimum amount of homology needed for integration of DNA fragments into the genome of yeast, DNA fragments were created by PCR amplification that contained the *URA3* gene flanked by different amounts of homology (5–90 bp) to the *LYS2* or the *ADE2* gene (Fig. 1). These fragments were produced by using primers that contained 5, 15, 25, 30, 45, 60, 75 or 90 bp of homology to the *LYS2* gene and 20 bp of sequence homologous to pBR322 flanking the *URA3* sequence to amplify *URA3*. For integration into the *ADE2* gene, the primers contained 25 or 30 bp homology to *ADE2* and 12 bp of homology to pBR322.

Strains RSY12 containing a complete deletion of the *URA3* fragment (ura3Δ; 5), RSY6 (*ura3-52*) and W303 (*ura3-1*) were transformed with the PCR products described above via a high efficiency transformation method (6,7). Integration can occur into *LYS2* by homologous integration or into sequences other than *LYS2* by illegitimate integration (5). These transformations yielded 2–14 transformants/μg of DNA. No net increase in the overall yield of transformants with increasing homology was found. Homologous integration into the *LYS2* gene results in lysine deficiency whereas illegitimate integration does not. To determine the fraction of homologous integration events we replica-plated ~200 colonies obtained by transformation with each of the different amplification products (modules) onto medium lacking lysine (Table 1). To our surprise, when we transformed LYS2-B which contains 15 bp of homology on each side of the fragment we obtained 3.6% lysine-deficient colonies (Table 1). When we used 25 bp of homology on each side (LYS2-C) we obtained 4.1% lysine-deficient colonies. This frequency jumped to 54% with 30 bp of homology on each side (LYS2-D, Table 1). With longer homology (LYS2-E to LYS2-H, Table 1) ~80% of lysine deficient colonies were obtained. To compare the above results with integration events in the presence of extended homology we used a *URA3* fragment flanked by 672 bp of *LYS2* sequences on one side and 1200 bp on the other side. Similar to the experiments with limited homology starting at 45 bp 80% of the colonies obtained were lysine deficient (LYS2-J102, Table 1). However, the extended homology caused an increase of 100-fold in the yield to 1000 transformants/μg.

Table 1. Integration of *URA3* fragments flanked by different amounts of homology to *LYS2* or *ADE2* in *ura3*Δ (RSY12) and *ura3-1* (W303) mutants

Module	Homology (bp)	Homologous integration (% *Lys*⁻, *Ura*⁺)[a]	
		RSY12 (ura3Δ	W303 (ura3-1)
LYS2-A	5	0.0 ± 0.0 (246)[b]	0.0 ± 0.0 (321)[b]
LYS2-B	15	3.6 ± 0.3 (272)	0.4 ± 0.1 (386)
LYS2-C	25	4.1 ± 0.2 (249)	1.4 ± 0.4 (216)
LYS2-D	30	53.9 ± 3.5 (63)	24.2 ± 2.3 (71)
LYS2-E	45	81.3 ± 5.0 (228)	34.2 ± 1.9 (282)
LYS2-F	60	95.6 ± 4.0 (234)	52.1 ± 1.7 (308)
LYS2-G	75	89.7 ± 3.4 (219)	36.6 ± 1.8 (327)
LYS2-H	90	88.9 ± 3.6 (147)	35.3 ± 2.5 (188)
LYS2-J102	672–1200[c]	80.3 ± 6.5 (860)	83.9 ± 9.0 (615)
ADE2-A	25	3.7 ± 0.5 (232)	0.8 ± 0.4 (460)
ADE2-B	30	70.5 ± 0.7 (68)	29.7 ± 3.0 (75)

Yeast strain RSY12 (*Mata leu2-3,112 his3-11,15 ura3::HIS3* designated *ura3*Δ) (5) was used. Yeast strain RSY6 (*Mata leu2-3,112 ura3-52 trp5-27 arg4-3 ade2-40 ilv-92 HIS3*::pRS6) contains the *ura3-52* mutation and BW303 (*Mata ade2-1 his3-11,15 leu2-3 trp1-1 can1-100 ura3-1 trp1-1*) contains a point mutation in the *ura3* gene and was obtained from Rodney Rothstein. Transformations with limited homology (5–90 bp) yielded 2–14 transformants/μg of DNA and transformations with the extended homology (672 and 1200 bp) yielded 10^3 to 10^4 transformants/μg of DNA.

[a]Mean values of three or more experiments.

[b]The number of transformants analyzed (parentheses).

[c]Amount of homology is 672 bp on one side and 1200 bp on the other side.

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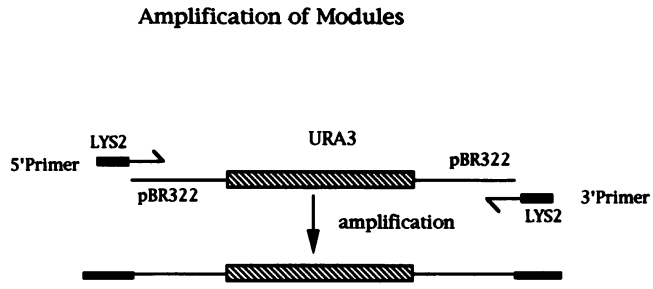

Figure 1. Plasmid PM53 was used as a template plasmid for PCR amplification and was constructed by insertion of a 1.5 kb *Bam*HI–*Eco*RI fragment containing *URA3* and pBR322 flanking sequences from the plasmid YEP24 into the pUC multicloning site. Plasmid pJZ102 contains extensive homology to *LYS2* and was used to disrupt the *LYS2* gene with *URA3* (8). Two primers (5′ primer and 3′ primer) were used for each amplification. Each primer contains sequences homologous to *LYS2* or *ADE2* as well as sequences homologous to the plasmid for amplification. The sequences underlined are complementary to the plasmid sequences for initial priming and the full-length sequences homologous to *LYS2* or *ADF2* in bold. **GTGGATGGATTTGGCAAACACAGTTGATATAATTATCCATAATGGTGCGTTAGTTCACTGGGTTTATCCATATGCCAAATTGAGGGATCC** TCATGTTTGACAGCTTATCATC (*LYS2* 5′) **GTAGAAGTGGAGGAAACAAAGTCAAAGAACTTTGGCTTGCCGACGGCGGCTAAGCTCATAACATTGATAGTTGAAATAACATTTGGATCC**GGACGGGTGTGGTCGCCATG (*LYS2* 3′) **ATGGTATAGCAGTTACCCAAAGTGT** TCATGTTTGACA (*ADE2* 5′) **CGTCTCACTGGCTTGTTCCACAGGA** GGACGGGTGTGG (*ADE2* 3′). The amount of homology ranged from 5 to 90 bp to *LYS2* and 25 to 30 bp to *ADE2*. For example, the primers that have 5 bp of homology to the target contain the plasmid sequences (underlined) but only 5 bp of the *LYS2* sequence (bold). After amplification each module will contain *URA3* sequences in the middle flanked by DNA with different amounts of homology to *LYS2* or *ADE2* as shown in this figure. The amplification was done for 30 cycles with 100 ng of denatured PM53 template DNA, appropriate primers, and VENT DNA polymerase (New England Biolabs). The amplification started with initial denaturation for 4 min at 95°C, the following cycles consisted of 10 s denaturation at 93°C, 30 s at 58°C, and 1 min extension at 72°C. The amplified DNA was precipitated with ethanol and suspended in TE.

We verified that the lysine deficient colonies contained the constructs integrated into the *LYS2* gene by Southern blot. Two lysine requiring colonies from transformations with each PCR product were chosen for Southern blotting. All lysine requiring colonies contained a *URA3* insertion into the *LYS2* gene for fragments hybridized to both *LYS2* and *URA3* probes (not shown) as expected for homologous integration. About 40 lysine prototroph colonies gave different sizes of fragments in agreement with being the result of illegitimate integrations (5) into positions other than *LYS2* (not shown).

To determine whether the results described above reflect a general phenomenon, we performed similar experiments with the gene flanked on each side by 25 or 30 bp of homology to *ADE2*. We found consistent results. With 25 bp of flanking homology, we obtained an average of 3.7% adenine deficient colonies. In contrast, when we used 30 bp of flanking homology, we obtained 71% of adenine requiring colonies (Table 1). Since the amount of homology is minimal compared to conventional integration events using extended homology, we termed these events in the presence of small homology 'microhomology mediated integration events'.

Other examples of successful targeting were provided by using primers containing 45 bp of homology to the *SUC2* gene to integrate FLAG epitope tagging fragments into the 3′ end of the *SUC2* gene (Weber and Schiestl, unpublished results), 35–51 bp of homology to different ORFs (2) and 35–50 bp of homology to *CNB1*, *FPR2* and *AUA1* genes (3). This method is currently widely used in other laboratories.

Most laboratory strains may not carry a complete deletion of *URA3*. Therefore, we investigated microhomology mediated integration events in strains RSY6 and W303 containing *ura3-52* and the *ura3-1* mutations, respectively. In these cases, the flanking homology may be degraded, and the *URA3* sequences from the fragments converts the *ura3* mutations to the wild-type allele. With strain W303 in the presence of 15 bp of flanking homology we obtained ~10% and with 25 bp ~30% of the number of targeted events that we obtained with the *ura3* deletion strain (Table 1). For the rest of the modules we found ~50% of targeted events except for the module containing the extended homology in which case the same percentage of targeted events (~80%) was found (Table 1). This result would be expected if gene conversion with the *ura3* locus would compete with integrations at *LYS2*. We saw the same trends for *ADE2* integrations. The same overall results have been obtained with strain RSY6 containing *ura3-52*.

In summary, we have defined the minimum amount of homology required for efficient homologous integration in *S.cerevisiae*. Homology of 30 bp on each side of a selectable marker is sufficient to obtain a large fraction of targeted integration events. This information can be applied to the economical design of primers for yeast genome modification by microhomology mediated PCR targeting.

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